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Detection, identification and semi-quantification of malaria parasites by NASBA amplification of small subunit ribosomal RNA sequences

Henk L. Smits[a,*], George C. Gussenhoven[a], Wiepko Terpstra[a], Rianne A.F. Schukkink[b], Bob van Gemen[b], Tom van Gool[c]

[a]*Department of Biomedical Research, Royal Tropical Institute, Meibergdreef 39, 1105 AZ Amsterdam, Netherlands*
[b]*Organon Teknika, Boseind 15, 5281 RM Boxtel, Netherlands*
[c]*Laboratory for Parasitology, Department of Medical Microbiology, University of Amsterdam, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, Netherlands*

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Abstract

We have adapted the Nucleic Acid Sequence Based Amplification method (NASBA), a molecular detection method for the amplification of specific RNA sequences, for the detection, identification and semi-quantification of malaria parasites. Primers and probes were selected based on the nucleotide sequence of the small subunit ribosomal RNA gene. NASBA enabled the detection of as little as 0.04 parasitized erythrocytes per μl blood. When applied to blood samples of patients, NASBA allowed the detection of malaria parasites in confirmed malaria patients. In addition, malaria parasites were detected in a number of patients with a history of suspected malaria. Semi-quantification over a wide parasite range was achieved by analysing serial end-point dilutions of RNA preparations. Semi-quantitative NASBA analysis of malaria parasites in follow-up samples of treated malaria patients showed a rapid decline of parasite levels in most patients and a low level of persistent parasites in some of the patients. © 1997 Elsevier Science B.V. All rights reserved.

Keywords: Malaria; Molecular detection; NASBA; RNA amplification; Quantification; Parasitemia

1. Introduction

In recent years malaria control programs have faced a world-wide increase in the number of malaria patients and of drug resistance. To apply control measures appropriately, sensitive diagnostic procedures are needed to identify foci of transmission and to monitor drug-treatment efficacy. Several microscopic [1], antigen detection [2] and molecular approaches [3,4] have been used for the detection of malaria parasites for diagnostic or epidemiological purposes. Of the direct detection methods, the polymerase chain reaction (PCR) is the most sensitive. Another amplification method, termed Nucleic Acid Sequenced Based Amplification (NASBA) [5–7], for the amplification of specific RNA sequences has not yet been applied for the detection of malaria parasites. Originally NASBA was developed for the detection of HIV-1 RNA [5]. Other studies have shown that NASBA can also be applied for the detection of mycobacteria in sputum [8,9] and for the

*Corresponding author. Tel.: +31 20 5665441; fax: +31 20 6971841.

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detection of human papillomavirus RNA in cervical smears [10]. These studies have shown that NASBA is a highly sensitive method allowing the amplification of as little as 10 to 100 target molecules. In addition NASBA can be used for the accurate quantification of RNA levels. With NASBA, quantification can be achieved over a wide range of target molecules [11,12]. Quantitative analysis of RNA levels after drug treatment could be a useful method to analyze drug-treatment efficacy in malaria patients. The applicability of NASBA for the determination of drug-treatment efficacy has been demonstrated for mycobacterial [13] and HIV-1 [14] infections.

Here, we describe the use of the NASBA technique for the detection, species identification and semi-quantification of malaria parasites. We selected a segment of the RNA of the small subunit ribosomal RNA gene (rrs) as target. For the amplification of the RNA of the rrs gene (ssurRNA) of *P. falciparum*, *P. vivax* and *P. malariae*, two general primers based on conserved regions within the plasmodium rrs genes but heterologous to the human rrs gene were chosen. The fragment of the rRNA amplified by the primer set contains a conserved region as well as a variable region suitable for detection and for species identification by appropriate probes. Semi- quantification of parasites was achieved by performing NASBA on ten-fold serial end-point dilutions of RNA preparations. Finally, the value of NASBA was evaluated on blood samples of suspected and treated malaria patients.

2. Materials and methods

2.1. Parasites

A culture of *P. falciparum* (strain Ghana) parasites was kindly provided by L. Hendrikx, Prince Leopold Institute of Tropical Medicine, Belgium. Parasites were cultured at 1% haematocrit in 5 ml RPMI-1640 medium (pH 7.2) supplemented with 10% pooled human serum, 0.2% $NaHCO_3$, 25 mM Hepes, 0.37 mM hypoxanthin and 11 mM glucose at 37°C. Cultures were aerated with 3% CO_2, 1% O_2 and 96% N_2. Parasites were harvested by centrifugation and collected in 5 ml of a mixture of 50% serum and 50% red blood cells.

2.2. Preparation and storage of samples

Fifty to 100 μl culture or blood samples were collected in 0.9 ml lysis buffer (5.25 M guanidinium isothiocyanate [GITC], 1.3% Triton X-100, 50 mM Tris–HCl pH 6.4, 20 mM EDTA) and mixed thoroughly. Lysed samples may be stored at −20°C for at least 2 weeks. For long term storage samples were kept at −70°C.

2.3. Total RNA isolation

Nucleic acids were isolated according to the GITC-silica procedure [15]. To the lysed samples, 40 μl of a sterile suspension of activated silica (coarse) in 0.1 M HCl was added. The lysate and the silica were mixed vigorously for 15 s and incubated for 15 min. The silica and bound nucleic acids were collected by a 15-s centrifugation step, the pellet was washed twice by thorough mixing and centrifugation with 0.7 ml wash buffer (5.25 M GITC, 50 mM Tris–HCl pH 6.4), twice with 0.7 ml 70% ethanol and once with 0.7 ml acetone. After removal of the acetone, the silica was dried by incubation at 56°C for 5 min. The dried silica was mixed with 100 μl RNAase free H_2O (Baker analyzed high-performance liquid chromatography reagent grade) and nucleic acids were eluted from the silica by incubation at 56°C for 15 min. Finally, the silica was pelleted by centrifugation and the supernatant was transferred to a fresh tube. To remove any remaining silica the tube was centrifuged again just before adding the sample to the reaction tube. During the extraction procedure, strict preventive measures were taken to avoid sample to sample contamination. Contamination was monitored by the inclusion of appropriate negative controls which were subjected to the complete extraction procedure. Powderless gloves were used to prevent inhibition of NASBA reactions by traces of powder. Waste and spills of GITC were treated with 1 M NaOH.

2.4. Selection of primers and probes

Primers and probes were selected based on the published sequences of the rrs genes of *P. falciparum* [16], *P. vivax* [17] and *P. malariae* [18], and of the human rrs gene. An alignment of the amplified region of the genome of the rrs gene of *P. falciparum*

with the corresponding sequences of the rrs genes of *P. malariae* and *P. vivax* along with the location of the different primers and probes used in this study is presented in Table 1. The downstream primer *P.*–P1 is completely homologous to the non-transcribed strand of the rrs genes of *P. falciparum*, *P. vivax* and *P. malariae* and the annealing site is located between positions 1236 and 1216 of the rrs gene [16]. The complete sequence of primer *P.*–P1 is 5'AATTCTAATACGACTCACTATAGGGAGAAG AATTTCTCTCGCTTGCGCGCGAA3' with the T7 promoter sequence underlined. The nucleotide sequence of primer *P.*–P2 is 5'TCAGATACGG-TCGTAATCTTA3'. The annealing site of *P.*–P2 is located between positions 1062 and 1082 of the transcribed strand of the rrs gene [16]. *P.*–P1 and *P.*–P2 show complete homology with their annealing sites on the ssurRNA *P. falciparum*, of *P. malariae* and *P. vivax*. *P.*–P1 and *P.*–P2 facilitate the amplification of a fragment of 174 bases of the *P. falciparum rrs* gene, a fragment of 183 bases of the *P. malariae rrs* genes and of a fragment of 189 bases of the *P. vivax rrs* gene. *P.*–P1 and *P.*–P2 show 9, respectively, 5 mismatches with the corresponding positions on the human rrs gene. A conserved region contained within the amplified segment was used to select a general probe (Pr–*P.*gen) with 100% homology to the hybridization site in the rrs gene of each of the plasmodium species. The sequence of Pr–*P.*gen is 5'CCTTATGAGAAATCAAAGTCTTT3'. Pr–*P.*gen shows 5 mismatches with the human gene (data not shown). An heterologous region was used for the selection of species-specific probes (Pr–*P.f*, Pr–*P.v* and Pr–*P.m*). This heterologous region is largely absent from the human gene. The nucleotide sequences of Pr–*P.f*, Pr–*P.m* and Pr–*P.v* are 5'AAAAGTCATCTTTCGAGGTGACTTT3', 5'GA-GACATTCTTATATATGAGTGTTTC3' and 5'AG-AATTTTCTCTTCGGAGTTTATTC3', respectively. *P. falciparum* contains two rrs genes, an asexually and a sexually expressed gene. The primers and probes described above were based on the sequences of the asexual gene. The primers *P.*–P1 and *P.*–P2, and the general probe Pr–*P.*gen also show complete homology with the sexually expressed rrs gene of *P. falciparum* [16] and thus potentially could be used to amplify and detect the sexually expressed ribosomal RNA. Compared with the annealing sites of Pr–*P.f* on the asexually expressed rrs gene the sequence of the sexually expressed rrs gene shows 10 mismatches and two insertions of 7, respectively 8 nucleotides.

Primers were purified by polyacrylamide gel electrophoresis before use.

2.5. In vitro transcription

The complete rrs genes of *P. falciparum*, *P. vivax* and *P. malariae* were kindly provided by Drs. A. Water and T.F. McCutchan. After PCR amplification of the genes, the genes were recloned as *Eco*RI–*Bam*HI fragments into pGem7 to facilitate transcription from the T7 promoter of the plasmid. Plasmids linearized with *Bam*HI were used for T7 RNA polymerase driven RNA synthesis (Boehringer). The RNA product was digested with RNAase free

Table 1
Primers and probes[a] used for the amplification, detection and identification of malaria parasites

Pf	5'TCAGATACCGTCGTCGTAATCTTAACCATAAACTATACCGACTAGGTGTTGGATGAAAGTG
Pm	. . . . . G . . . . . T . . . .
Pv	. . . . . CT . . . . . AT
Pf	TTAAAAATAAAAGTCATCTTTCGAGGTGACTTT- - - - - - - - TAGATTGCTTCTTTCAGTA
Pm	. A . . . . . GAGACATTCTTATATATGAGTGTTTC TTT . . . . . A . . . . .
Pv	. . T . . . . . AGAATTTTCTCTTCGGAGTTTATTC- - - - - - T . . . . . G
Pf	*CCTTATGAGAAATCAAAGTCTTT*GGGTTCTGGGGCGAGTATTCGCGCAAGCGAGAAAGTT3'
Pm	. . . . .
Pv	. . . . .

[a] *Pf*, *Pm* and *Pv* represent the amplified regions of the ssurRNA of *P. falciparum*, *P. malariae* and *P. vivax* respectively. The region used for the selection of the primers *P.*–P1 and *P.*–P2 are underlined with a single line, the species specific probes Pr–*P.f*, Pr–*P.v* and Pr–*P.m* are underlined with a double line, and the general probe Pr–*P.gen* is presented in italics. Dots indicate nucleotide identity and dashes indicate the lack of corresponding nucleotides.

DNAase, extracted with phenol/chloroform and, after ethanol precipitation, dissolved in sterile H_2O (Baker).

2.6. NASBA

The NASBA reactions were performed as described by Kievits et al. [5] with some modifications. Firstly, a 21-μl volume reaction mixture was prepared by mixing 4.75 μl H_2O, 10 μl of a buffer consisting of 40 mM Tris–HCl pH 8.5, 12 mM $MgCl_2$, 42 mM KCl, 5 mM DTT, 1 mM of each dNTP and 2 mM of each rNTP, and 6.25 μl of a primer mix consisting of 60% v/v DMSO and 0.8 μm of each of the two primers. After the addition of 2 μl of target RNA, the test tubes were incubated for exactly 4 min at 65°C in order to melt the RNA after which the tubes were quickly transferred to 41°C. After a 4 min incubation at 41°C, 1.7 μl of enzyme mixture was added, containing 0.1 μg BSA (Boehringer), 38 U T7 RNA polymerase (Pharmacia), 8 U AMV–RT (Seikagaku) and 0.1 U RNAase H (Pharmacia). Isothermal amplification of the RNA target was performed by incubation for 2 h at 41°C. NASBA products were stored at −20°C. The reaction has been optimized using different primer combinations and by using different KCL concentrations by performing the reaction on tenfold serial dilutions of in vitro synthesized *P.f*, *P.v* and *P.m* ssurRNA ranging in concentration from 2 to 2×10^6 molecules per reaction. Addition of ITP (0.5 mM rITP plus 1.5 mM rGTP instead of 2.0 mM rGTP) [13] reduced the efficacy of amplification. With the conditions described, 200 ssurRNA molecules from each of the three malaria species could be detected, on average, after hybridization with either the general probe or the corresponding species-specific probe (data not shown). In most NASBA reactions two products both hybridizing to the probe were observed. We speculate that these two products represent the same molecule but with a different secondary or tertiary structure or that one of the intermediates of the NASBA amplification cycle [5–7] has accumulated as well. To prevent product contamination, RNA isolation, assembly of the reaction mixture, initiation of the amplification reaction and product analysis were each done in different laboratories.

2.7. Semi-quantitative NASBA

Semi-quantification of malaria parasite RNA was performed by applying NASBA to serial tenfold dilutions of RNA prepared in 50 μl H_2O. For the validation of the assay, serial tenfold dilutions of cultured parasites were prepared in 100 μl of a mixture of 50% serum and 50% red blood cells and RNA was prepared from each dilution.

2.8. Gel electrophoresis

Six μl of the NASBA product was mixed with 3 μl layer mix (0.25% bromophenol blue and 0.25% xylene cyanol in 15% Ficoll Type 400) and run in 1×TAE buffer (10×TAE=0.4 M Tris–acetate and 0.01 M EDTA) on a 2% agarose gel containing 3 μg ml^{-1} ethidium bromide.

2.9. Blotting procedure

After electrophoretic separation, products were allowed to transfer for 90 min in 2×SSC (1×SSC is 0.15 M NaCl, 0.015 sodium citrate pH7.0) to a prewetted positively charged Nylon membrane (Boehringer) using a vacuum blot apparatus. After completion of the transfer the RNA was fixed to the membrane by UV illumination (1 J cm^{-2}).

2.10. Hybridization

Filters were prehybridized for 30 min and hybridized to digoxigenin (DIG)-labelled probes (5 pmol/ml) in 6×SSC, 20 mM sodium phosphate pH 6.7, 2% SDS and 10×Denhardt's (1×Denhardt's=0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA) for 18 h. To account for the differences in melting temperature (Tm), hybridization was performed at 52°C for the general probe and at 42°C for the species-specific probes, and hybridized filters were washed thrice for 3 min at room temperature in 0.3×SSC, 0.1% SDS for the general probe and in 3×SSC, 1% SDS for the species-specific probes. DIG labelling of probes by the DIG oligonucleotide tailing kit and chemiluminescent detection of washed

filters with anti-digoxigenin conjugated phosphatase and Lumigen PPD was performed as recommended by the manufacturer (Boehringer). Blots were exposed to X-rays for 60 min.

2.11. Enzyme-linked gel electrophoresis assay (ELGA)

For detection of NASBA products by the enzyme-linked gel electrophoresis assay [8] 3 μl of the NASBA product was mixed with 3 μl of probe mixture consisting of 1 μl 6×SSC, 1 μl layermix (0.25% bromophenol blue and 0.25% xylene cyanol in 15% Ficoll Type 400) and 1 μl horseradish peroxidase labelled detection probe at a concentration of 0.8×10^{10} μmol μl^{-1}. After the addition of the probe mixture, the NASBA product was allowed to hybridize to the labelled probe by incubation at 41°C for 15 min. After hybridization 2.5 μl was run on a 7% polyacrylamide–dextran sulfate gel in 0.5× TBE (1×TBE=0.089 M Tris–borate, 0.89 M boric acid and 0.002 M EDTA) for 1 h at 100 V. The gel was prepared by mixing 1.85 ml 39% acrylamide and 1% bisacrylamide, 0.25 ml 10×TBE, 0.4 ml 1% dextran sulfate, 6.25 ml distilled H_2O, 100 μl 30% ammonium persulfate and 10 μl *N,N,N′,N′*-tetramethylethylenediamine. After electrophoresis the gel was stained by incubation in 24.5 ml 1×TMB buffer (10×TMB buffer=100 mM citrate, 100 mM EDTA pH 4.8), 0.5 ml 50×TMB substrate (50×TMB substrate=20 mg 3,3′,5,5′-tetramethylbenzidine in 4 ml dimethylsulfoxide) and 2.5 μl 30% H_2O_2 at room temperature. Results were recorded after 10 min. Gels stained for 18 h were fixed in 50% methanol and dried on a KEN EN TEC gel drying frame.

2.12. Patients

From 125 patients suspected of malaria, a total of 200 finger prick blood samples were collected. Thin smear, thick smear and QBC were used for the detection of parasites and species determination. Malaria parasites were detected in 24 patients. All 24 malaria patients received prompt treatment. Follow-up samples were available for analysis for 12 of the malaria patients.

3. Results

3.1. Detection limit of NASBA

The detection limit of NASBA for plasmodium parasites was determined on RNA extracted from a tenfold serial dilution of cultured *P. falciparum* parasites (4×10^3 parasitized erythrocytes μl^{-1}). As little as 4 parasitized erythrocytes per 100 μl sample (0.04 parasitized erythrocytes μl^{-1}) could be detected (Fig. 1). Comparison of the signal strength at low and high target levels showed that an almost maximal signal strength was obtained at the lowest

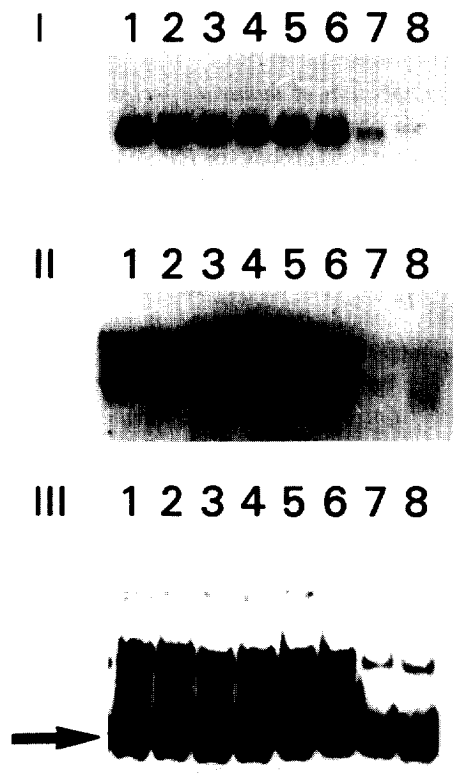

Fig. 1. Detection limit of NASBA for malaria. A tenfold serial dilution of 100 μl cultured parasites (*P.falciparum*; 4×10^3 parasitized erythrocytes μl^{-1}) was prepared in 100 μl of a mixture of 50% serum and 50% red blood cells. Total nucleic acids were prepared from each dilution and dissolved in 100 μl; the equivalent (2 μl) of 8000 (lane 1), 800 (2), 80 (3), 8 (4), 0.8 (5), 0.08 (6), 0.008 (7) and 0.0008 (8) parasitized erythrocytes was used as target. I, Ethidium bromide stained gel; II, Northern blot hybridized to the general probe Pr–*P.*gen; III, ELGA hybridized to Pr–*P.*gen. The position of the free-probe is indicated (arrow).

detectable target level. Confirmation of the identity of the NASBA product by hybridization however is required as, in negative control reactions (using RNA prepared from nonparasitized erythrocytes), an aspecific product which migrated slightly faster than the specific product and which clearly did not hybridize with the probe was visible on the ethidium bromide stained gel. To determine whether serum and red blood cells might influence the efficiency of NASBA, the dilutions were also prepared in a mixture of 50% serum and 50% red blood cells as RPMI. The presence of serum and red blood cells did not affect the detection limit (data not shown). These results indicate that NASBA may allow the detection of parasites in blood samples of patients with a parasitemia as low as 10^{-6}%.

3.2. Identification of malaria species

Species-specific probes for *P. falciparum* (Pr–*P.f*), *P. vivax* (Pr–*P.v*) and *P. malariae* (Pr–*P.m*) were selected based on a non-conserved region within the ssurRNA segment spanned by the two amplification primers. These species-specific probes appeared to be highly specific as even at a high target concentration (2×10^5 molecules) no cross-reactivity was observed (Fig. 2). Also the NASBA product of RNA prepared from cultured *P. falciparum* parasites was specifically detected with the *P. falciparum*-specific probe (Fig. 2).

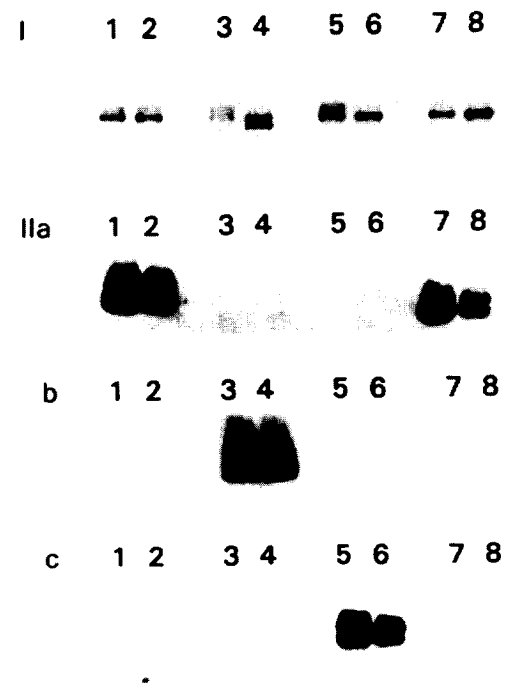

Fig. 2. Species specificity of the species-specific probes. NASBA was performed on 2×10^5 (lanes 1, 3 and 5) and 2×10^3 (2, 4 and 6) molecules of in vitro synthesized *P. falciparum* (lanes 1 and 2), *P. vivax* (3 and 4) and *P. malariae* (5 and 6) ssurRNA, and on RNA extracted from 10^4 (lane 7) and 10 (8) cultured *P. falciparum* parasites. I, Ethidium bromide stained gel; II, Northern blots hybridized with the species-specific probes Pr–*P.f* (a), Pr–*P.v* (b) and Pr–*P.m* (c).

3.3. Visualization of the NASBA result by ELGA

Application of ELGA for the detection of the NASBA product of a tenfold serial dilution of cultured *P. falciparum* parasites revealed that with ELGA the same sensitivity is reached as with Northern blot hybridization (Fig. 1). The suitability of ELGA as a detection method was investigated as this method is much faster and easier to perform than Northern blotting.

3.4. Detection of malaria parasites in blood samples of suspected malaria patients

We examined 200 blood samples from 125 patients suspected of having malaria. Malaria parasites were detected by the combined results of thin smear, thick smear and QBC in 38 blood samples from 24 patients. NASBA was applied to each of the 200 blood samples and to 150 negative control samples prepared from blood obtained from the local blood bank. The negative control samples were analyzed in between the patients' samples and served to monitor contamination. Northern blot hybridization and ELGA were used to detect NASBA positive samples. An ethidium bromide stained gel, a Northern blot and an ELGA showing some of the results are presented in Fig. 3. Northern blot hybridization and ELGA showed agreement in all but one of the samples.

NASBA was positive in 37 out of the 38 samples in which parasites were observed by microscopy (Table 2). All but one of the 24 malaria patients were found positive by NASBA. A relatively high parasite count (2.8%) was determined for the false

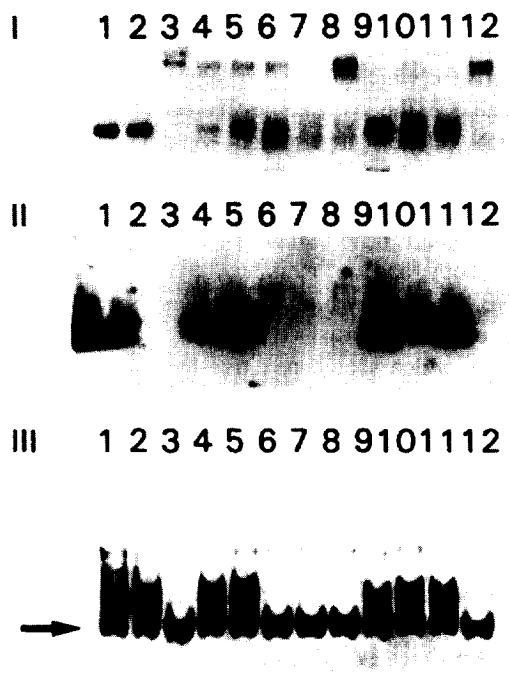

Fig. 3. Detection of malaria parasites in human blood samples. NASBA results of 12 blood samples (lanes 1–12) are presented. I, Ethidium bromide stained gel; II, Northern blot hybridized with Pr–*P.*gen; III, ELGA hybridized with Pr–*P.*gen. The position of the free-probe is indicated (arrow).

negative result. Ninety-one patients (131 samples) were judged negative by microscopy and by NASBA. NASBA was positive in 31 parasite-negative samples. Ten of these 31 samples were from 10 patients with a history consistent with past or mild malaria, and 21 samples were follow-up samples from patients under treatment.

Three of the ten NASBA positive patients with a history consistent with malaria had been diagnosed recently for malaria at a different hospital and had received treatment. The other seven patients were recent immigrants from a tropical country in which malaria is highly endemic. We cannot completely exclude the possibility that these samples had been contaminated. However, of the 150 negative control samples only two were contaminated during the sample preparation procedure. One of these two samples was positioned, during the procedure, adjacent to a strongly positive blood sample.

Table 2
Detection of malaria parasites by microscopy and NASBA

Total number of samples	Microscopy positive /NASBA positive	Microscopy positive /NASBA negative	Microscopy negative /NASBA positive	Microscopy negative /NASBA negative
200	37	1	31	131

Follow-up analysis of 12 treated malaria patients showed that parasites became undetectable by microscopy 2 to 5 days after the start of treatment (data not shown). However, from 11 of these 12 patients, blood samples remained NASBA positive for several days to weeks after microscopical clearance of parasites from the blood. One of the patients showed recrudescence at day 26 and the reappearance of parasites in the blood also was detected by NASBA. In another patient NASBA became positive again at day 18, 2 weeks after the last NASBA and microscopically positive sample. In this patient no parasites were seen by routine microscopical observation of the blood sample taken at day 18.

3.5. Species identification by NASBA

Identification of the species was possible by microscopy in 21 of the 24 proven malaria patients. Of the 21 patients, 18 were infected with *P.falciparum* and three with *P. vivax*. Species identification by hybridization of the NASBA product with the species-specific probes was possible in all patients. NASBA indicated a *P. falciparum* infection in the three patients in which the species could not identified by microscopy. The results of microscopy and NASBA disagreed for one sample in which microscopy showed *P.falciparum* and NASBA indicated *P. vivax*. An example of the hybridization of the NASBA product of patient samples is presented in Fig. 4. The presented samples all hybridized to the *P. falciparum* specific probe.

3.6. Semi-quantification of malaria parasites in blood samples through the amplification of RNA

To analyze the kinetics of parasite clearance in treated malaria patients, a semi-quantitative NASBA assay was developed and applied to each of the positive NASBA samples. The assay was first validated in a reconstitution experiment using tenfold serial dilutions of RNA prepared from known

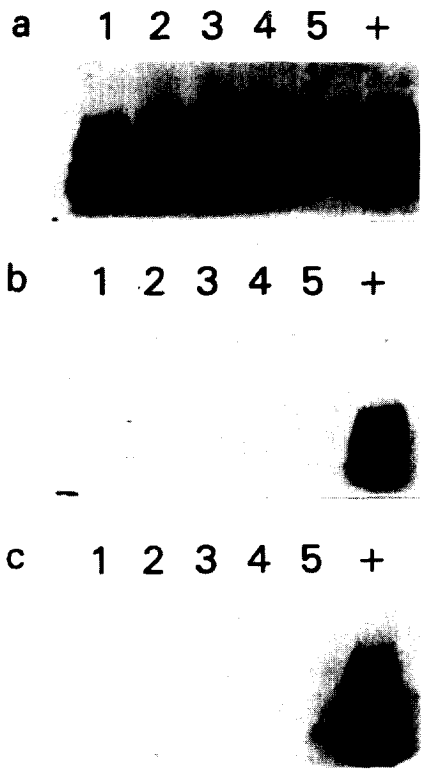

Fig. 4. Species identification by NASBA on blood samples. The NASBA products of the five NASBA positive samples (lanes 1–5) were blotted and hybridized with probes Pr–*P.f* (a), Pr–*P.v* (b) and Pr–*P.m* (c). As a positive control (lanes marked +) NASBA was performed on in vitro synthesized ssurRNA of *P. falciparum* (a), *P. vivax* (b) and *P. malariae* (c).

amounts of cultured parasites. In this experiment RNA was prepared from 8 tenfold serial dilutions of cultured parasites with 40 000 parasitized erythrocytes μl^{-1} as the highest parasite concentration and NASBA was performed on tenfold serial dilutions prepared from each of these RNA preparations. The experiment showed a linear relationship between the highest RNA dilution still positive and the number of parasites present in the original parasite sample such that a highest positive RNA dilution of 10^{-6} corresponded to a parasite level of 40 000 parasitized erythrocytes μl^{-1} and the original RNA sample as the highest positive dilution to a level of 0.04 parasitized erythrocytes μl^{-1}. In different experiments the observed highest positive dilution was at the most one dilution step higher or lower than the expected highest positive dilution.

The semi-quantitative NASBA assay was applied to each of the blood samples of the 12 treated patients for which follow-up samples were available. In Table 3 the parasite levels calculated from the ssurRNA levels determined for these samples is compared with the microscopical observation of parasites. The results show that most samples (21 out of 24 samples) in which parasites were seen by microscopy had, as estimated by semi-quantitative NASBA, relatively high parasite levels of 40 parasitized erythrocytes μl^{-1} or more, and that most samples which scored negative by microscopy were either NASBA negative (12 samples) or (18 out of 21 NASBA positive samples) had a low parasite level of 4 to 0.04 parasitized erythrocytes μl^{-1} only. This result is in good agreement with the known detection limit of microscopy of 1–20 parasite μl^{-1}.

The results of the semi-quantitative NASBA assay are graphically presented in Fig. 5 for the follow-up samples from one of the 12 patients. In this patient the parasite level dropped, as determined by NASBA, to around the detection limit of microscopy at the time (day 3) when the blood sample became microscopically negative, and then further dropped to 0.4 parasitized erythrocytes μl^{-1} at day 7 before NASBA became negative at day 13. In six other patients the kinetics of parasite clearance showed a similar pattern of an initial rapid drop in parasite level followed by a persistent low level of parasites. In three of these six patients low parasite levels persisted for 2 to 3 weeks. In five other patients the kinetics of parasite clearance could not be deter-

Table 3
Correlation of parasite levels and microscopy of follow-up samples of treated patients

NASBA		Microscopy	
Highest positive 10-fold dilution	Calculated parasitized erythrocytes μl^{-1}	Number of positive samples	Number of negative samples
10^{-5}	≥4000	5	0
10^{-4}	400	8	2
10^{-3}	40	8	1
10^{-2}	4	1	8
10^{-1}	0.4	2	5
1	0.04	0	5
Negative	<0.04	0	12

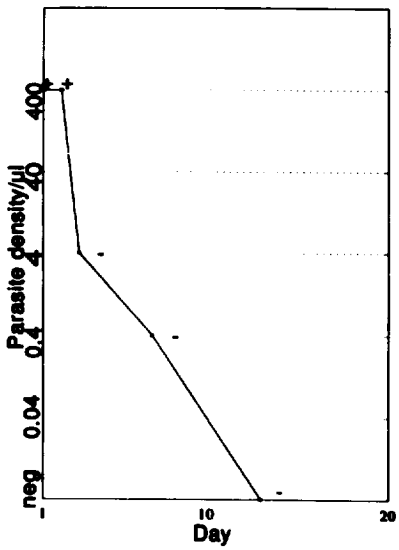

Fig. 5. Example of parasite clearance as measured by semi-quantitative NASBA. The parasite level was determined by NASBA analysis of six tenfold serial RNA dilutions. The microscopical results (+, positive; −, negative) are indicated.

mined accurately due to lack of sufficient follow-up samples within the first week after treatment. In three of these five patients follow-up samples taken between 2 to 4 weeks after treatment were negative or indicated low parasite levels only. In the other two patients follow up samples were limited to day 2 and 3. In the one patient with microscopical reappearance of parasites at day 26, a parasite level of 4 parasitized erythrocyte μl^{-1} was detected with NASBA at the same day and again 1 week later. The NASBA results of another patient suggested recrudescence while microscopy remained negative. In this patient strongly positive NASBA signals at day 1 and 4 were followed by a NASBA negative sample at day 11 and again positive samples (4–0.4 parasitized erythrocytes μl^{-1}) at days 18, 25, 27 and 34.

4. Discussion

NASBA applied on a segment of the ssurRNA of malaria parasites allowed the detection of the four parasites in 100 µl blood or 0.04 parasites per µl. Microscopy has a detection limit of 1–20 parasites per µl. The use of species-specific probes allows the identification of *P. falciparum*, *P. vivax* and *P. malariae*. A specific probe for *P. ovale* may become available once the complete nucleotide sequence of the rrs gene of this species is known. Using the primer set described, NASBA potentially also may be used to discriminate between the expression of the asexually and the sexually expressed rrs gene of plasmodium. The annealing site of the two primers used for amplification is completely homologous between the two genes of *P. falciparum* and the region of the genes spanned by the two primers shows sufficient heterology to allow selection of specific probes.

The detection of the NASBA product requires hybridization as non-specific products are formed in negative control reactions (Fig. 3). This disadvantage was partly compensated for by the performance of hybridization by ELGA [8], a method which is relatively easy and quick to perform. In ELGA a horseradish peroxidase conjugated probe is hybridized to 3 µl of the NASBA product. The hybridization product is then run directly on a polyacrylamide gel and the result is visualized by incubation of the gel in the appropriate substrate. The complete procedure takes about 2 h and this includes 15 min for hybridization, 60 min for gel electrophoresis and 10 min for staining of the gel.

An advantage of NASBA is that semi-quantification may be achieved over a wide range of target molecules. In a reconstitution experiment using a known number of cultured parasites, we demonstrated that semi-quantification over a wide range of parasitemia is possible. Semi-quantification was achieved by performing NASBA on tenfold serial dilutions of samples. The accuracy of quantification is limited by the tenfold dilution step and is probably influenced by variations in the sampling method, the RNA preparation method and the amplification reaction. The relatively large sample volume (75 µl) used for RNA extraction may compensate for an uneven distribution of parasites and thus increase the accuracy. Potentially, the accuracy of quantification could be increased by analyzing multiple replicates of serial dilutions. At the limit of dilution where only some reactions are positive, the number of targets may then be calculated from the proportion of negative reactions by using Poisson statistics. In addition, van Gemen and coworkers [10,11] demon-

strated that accurate quantification by NASBA can be achieved by co-amplification of a known amount of an internal standard and that by using multiple standards, each included in the reaction at a different concentration, quantification may even be performed with a single amplification reaction.

We applied NASBA for the detection of malaria parasites in blood samples from suspected malaria patients. The samples of all but one of the malaria patients were positive in NASBA. In addition, the samples of ten patients with a history of suspected malaria were found to be positive. Our results suggest that NASBA may well be used for accurate and sensitive epidemiologic measurement of malaria. The low detection limit of NASBA may make this method particularly suitable to identify foci of transmission and to identify patients for whom a low level of parasites is expected, such as pregnant women or partially treated patients.

Most patients became microscopically negative a few days after treatment. In contrast, analysis of the available follow-up samples by NASBA revealed a prolonged positivity for at least 1 or 2 weeks after treatment. These results suggested that low parasite levels may persist after treatment. This was further investigated by semi-quantitative NASBA analysis of the follow-up samples. The results showed a dramatic drop in parasite levels during the first few days of treatment from between 40 and 4000 parasites or more per μl blood to a level at or below the detection limit of microscopy at the time when microscopy became negative. Semi-quantitative NASBA analysis of the microscopically negative, NASBA positive follow-up samples showed that most of these samples contained parasite RNA levels corresponding to a low parasitemia of 4–0.04 parasitized erythrocytes per μl. The rapid drop of the parasite level shortly after the onset of treatment strongly argues against the possibility that the prolonged positivity of NASBA is explained solely by the persistence of killed parasites or of their RNA. Our results indicate that in treated patients, after an initial rapid decline of parasite numbers, low levels of parasites may persist for a considerable time.

The persistence of low levels of parasitic RNA in some treated patients could be the result of a slow removal of parasites from the internal organs but also may reflect a suboptimal treatment regimen in these patients. Such a suboptimal treatment efficacy could be the result of a low level of drug resistance of the parasites. In one of the patients treatment failure resulted in recrudescence of parasites. In this patient recrudescence was detected by microscopy as well as by NASBA. In a second patient NASBA results suggested recrudescence but this could not be confirmed by microscopy. Characterization of the drug sensitivity of parasites and the effects of different drug-treatment regimens on parasite levels will be required to determine whether quantification of parasite levels by NASBA could be used to estimate drug-treatment efficacy and to measure drug resistance. Another potential application of the semi-quantitative NASBA assay could be to monitor the efficacy of vaccination programs.

Several other studies have described semi-quantitative methods for the analysis of malaria parasite levels. In these studies either PCR [19] or RT-PCR [20,21] were being used. Advantages of the NASBA method described here could be that the RNA extraction method employed yields highly stable RNA preparations, that amplification does not requires a thermocycler, and that detection is achieved relatively quickly and requires no radioactive-labelled probe. Furthermore, as described elsewhere, semi-quantization potential can be achieved within a single tube with NASBA [11,12].

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